Mechanisms of drug-induced apoptosis in human leukemia

Cavagis, Alexandre Donizeti Martins

Chapter 3

Kinome profiling for uncovering the molecular mode of pharmacon action

Carmen Veríssima Ferreira[1,7], Alexandre D. Martins Cavagis[1], Sander H. Diks[2,7], Francesca Milano[3], Carina L. Bos[3], Giselle Z. Justo[1], Nelson Durán[4,5] and Maikel P. Peppelenbosch[2,6]

[1]*Departamento de Bioquímica, Universidade Estadual de Campinas São Paulo, Brazil*

[2]*Department of Cell Biology, Faculty of Medicine*

University of Groningen, A. Deusinglaan 1 NL-9713 AV Groningen, The Netherlands,

Tel: 31-50-363 2522, Fax: 31-50-363 2512

E mail : M.Peppelenbosch@med.rug.nl

[3]*Experimental Internal Medicine, Academic Medical Center*

Amsterdam, The Netherlands

[4]*Núcleo de Ciências Ambientais, Universidade de Mogi das Cruzes.*

São Paulo, Brazil

[5]*Biological Chemistry Laboratory, Instituto de Química,*

Universidade Estadual de Campinas

São Paulo, Brazil

[6]*To whom correspondence should be addressed*

[7]*Contributed equally to this work*

ABSTRACT

Defining the molecular effects of compounds with clinically useful properties remains exceedingly challenging if no *a priori* assumptions can be made as to the biochemical details of the biological effect observed. We set out to identify the molecular target of violacein, a purple-coloured pigment produced by the Amazon River *Chromobacterium violaceum*, which is used by indigenous Indians in the Amazon forest to treat a variety of inflammatory conditions and attracts substantial interest as a consequence of its anti-leukaemic properties. To this end, we compared lysates of violacein-treated cells and vehicle-treated cells for in vitro phosphorylation of peptide arrays containing 1152 different kinase consensus substrates. The results provide a wealth of data on violacein-dependent biochemical effects. From this kinome profiling effort, the p42/p44 MAP kinase pathway emerged as a major target for violacein. Subsequent studies revealed that activation of the p42/p44 MAP kinase pathway is essential for violacein-dependent effects, triggering differentiation of the leukaemia cells.

INTRODUCTION

Establishing the molecular mode of action compounds with clinically useful properties is difficult if there is no information as to the biochemical details of the biological effect observed. Over the last 5 years array and mass spectrometry technologies have enabled the determination of the transcriptome and proteome, and such information will likely be of significant value to our elucidation of the molecular mechanisms that govern the effects of pharmacological compounds. However, defining those proteins that participate in signalling pathways affected by such compounds may provide more direct insight into the mechanism underlying the clinical effects of such compounds. Enzymes that phosphorylate tyrosine, serine and threonine residues on other proteins play a major role in signalling cascades that determine cell cycle entry, survival and differentiation fate in the tissues of the mammalian body and traditional genetic and biochemical approaches can certainly provide answers as to the molecular details that underlie pharmacon action, but for technical and practical reasons there are typically pursued one gene or pathway at a time. Thus, a more comprehensive approach is needed in order to reveal all signalling pathways influenced by a pharmacological compound in a single experiment. Adaptation of array technology for measuring enzymatic activity in a parallel fashion seems a obvious solution and recently progress in this direction has been made with the preparation of protein chips for the assessment of protein substrate interactions [1–3] and the generation of peptide chips for the appraisal of ligand-receptor interactions and enzymatic activities.[4–7] Houseman and Mrksich[8] showed that peptide chips, prepared by the Diels-Alder-mediated immobilization of one kinase substrate for the non-receptor tyrosine kinase c-Src) on a monolayer of alkanethiolates on gold, allows quantitative evaluation of kinase activity. We recently showed that ^{33}P-γ-ATP phosphorylation of arrays consisting of 192 peptides (substrates for kinases) spotted on glass by cell lysates from human peripheral blood mononuclear cells allowed the simultaneous description of the temporal kinetics of a multitude of kinase activities following stimulation with lipopolysaccharide.[9] It appeared, however, that the amount of substrates on this array was insufficient to allow comprehensive descriptions of the effects of pharmacological intervention on cellular signal transduction.

This consideration prompted us to study the effectiveness of an array with substantially increased numbers of substrates for obtaining complete descriptions of the effect of pharmacological intervention on the kinome. To this end we selected a set substrates covering almost the entire mammalian kinome from the Phosphobase resource (http://phospho.elm.eu.org)[10,11] (a full list of the peptides and the proteins from which they are derived is listed in the supplementary data) and arrays were constructed arrays by chemically synthesizing soluble peptides, which were covalently coupled to glass substrates as described earlier. Arrays consisted of 1152 different nonapeptides, providing kinase substrate consensus sequences across the entire mammalian kinome. On each separate carrier, the array was spotted two times, to allow assessment of possible variability in substrate phosphorylation. The final physical dimensions of the array were 25 x 75 mm, each peptide spot having a diameter of approximately 250 µm, and peptide spots being 620 µm apart. Employing this design, we have recently been able to identify Fyn and Lck as kinase inhibited during glucocorticoid treatment of human peripheral blood lymphocytes.[12]

We decided to explore the effects of violacein (Figure 1a), a purple-colored pigment cum antibiotic produced by Amazon River Chromobacterium violaceum, which is used by indigenous Indians in the Amazon forest to treat a variety of inflammatory conditions and attracts substantial interest as a consequence of its anti-leukemic properties. Recently we showed that violacein is a member of a novel class of cytotoxic drugs mediating apoptosis of HL60 cells, probably via the induction of TNF receptor signal transduction.[13] When lysates of violacein-treated HL60 cells were investigated on Western blot, using anti-phospho-amino acid antibodies, numerous changes in global phosphorylation patterns are detected (Figure 1b) and thus it is reasonable to assume that at least some kinase activities are affected by the violacein treatment. Thus, employing the peptide array, the effect of violacein on the HL60 cell kinome we set out to generate a comprehensive description of violacein effect on leukaemia cell kinases.

STUDY DESIGN

Cell Culture

HL60 cells were grown in suspension in RPMI 1640 medium according to routine procedures.[13] Violacein dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium which had the final DMSO concentration adjusted to 0.1% (v/v).

Peptide arraying

The production of the array and the protocol of the kinome array have been described in detail earlier.[9,12] In short, cells were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan, Lelystad, the Netherlands), containing up to 1152 different kinase substrates in duplo, were incubated with cell lysates for 2 hours in a humidified stove at 37C°. Subsequently, the arrays were washed in 2M NaCl, 1% triton-x-100, PBS, 0.1% tween and H_2O, where after slides were exposed to a phospho-imaging screen for 24-72 hours and scanned on a phospho-imager (Fuji, Stamford, USA).

Western Blotting

Cells (3×10^7) were lysed in 200 µl of denaturing lysis buffer ans subjected to SDS-PAGE and Western blotting as described in detail previously.[12,13] Detection was performed using enhanced chemiluminescence (ECL).

Cell differentiation Assay

Control and violacein-treated cells were collected and resuspended in 1X binding buffer (0.01 M Hepes/NaOH, pH 7.4, 0.14 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Subsequently, 100 µl of cell suspension was transferred to a 5-ml tube

and CD11 FITC (5 µl) and CD66 - (10 µl) were added. The cells were incubated at room temperature for 15 minutes, after which 400 µl of 1X binding buffer was added, and differentiation analysed by flow cytometry.

Statistical evaluation

The western blots represent 3 independent experiments. Cell viability was expressed as the means ± standard errors of 3 independent experiments carried out in triplicate. Data for each assay were analysed statistically by ANOVA

RESULTS AND DISCUSSION

To investigate the effects of violacein on the kinome, 2.5 x 10^5 HL60 cells were incubated for 24 hrs with vehicle (DMSO) or violacein and subsequently analyzed using the peptide array. An example is shown in figure 2a. Subsequently, we analyzed the radio-activity incorporated in substrates phosphorylated by vehicle treated cells against the radio-activity incorporated in substrates phosphorylated by violacein-treated cells. The relationship between the two data-sets is roughly linear, suggesting that most cellular kinases are not directly influenced by the violacein treatment, in apparent agreement with its mild effect on primary non-transformed human leukocytes.[13] Nevertheless, interesting differences are observed, phosphorylation of 49 peptides being significantly different when violacein-treated and vehicle-treated HL60 cells were compared ($p<0.05$; see supplementary information for a full description of results obtained). These peptides include 2 substrates (#6 and #868 in the supplementary data) annotated as targets for c-fms proto-oncogene tyrosine kinase activity, which is an important regulator of myeloid cellular physiology. In addition, phosphorylation of peptides acting as substrates for the Src-like kinases c-Hck (#395), c-Lck (#361), and c-Src (#700 and #755) itself were substantially down regulated. Also increased phosphorylation of a peptide of a Jun-N-terminal kinase (JNK)-derived peptide that serves as a substrate of MKK 4/7 was observed, whereas also increased phosphorylation of p53. In addition, peptides that are substrates for p34cdc2 (#172, #461, #462, #643, #744, #904, and #1121) were less phosphorylated by cell lysates of violacein-treated cells. Thus violacein influences various cellular signalling systems that could be important for mediating its anti-leukaemic effect.

Strikingly, however, the most up-regulated substrate d substrate phosphorylation events occurred in peptides specific for p42/p44 MAP kinase (#181, #460, #625), Phosphorylation of a peptide specific for the upstream activator of MAPK, c-Raf (#863) was also highly up-regulated by the violacein treatment, suggesting activation of the MAPK signalling cassette in leukaemia cells by violacein and this notion was confirmed in Western blot experiments (figure 2c) employing phosphospecific antibodies. These experiments demonstrated enhanced immunoreactivity for pS_{338} c-

Raf, pS_{221}MEK and pT_{202}/pY_{204} p42/p44 MAP kinase, thus violacein stimulates the three major components of the MAP kinase signalling cassette. Concomitantly, immunoreactivity towards pT_{180}/pY_{182} was unchanged and thus the effects of violacein on the MAP kinase cascade are not the result of aspecific kinase activation although at the same time immunoreactivity for pT_{183}/pY_{185} JNK did increase, but this was confirm expectation from the peptide array results. Thus activation of the p42/p44 MAP kinase cascade is a prominent effect of violacein on cellular physiology.

Activation of the MAK kinase cascade by a anti-leukaemic compound was surprising, but this signalling pathway has been associated with leukaemic cell differentiation and cell cycle arrest.[14] Indeed, the down regulation of cdc2 enzymatic activity observed in the peptide array experiments suggests cell cycle arrest in response violacein and such cell cycle arrest was confirmed on Western blot employing an antiserum against the cell cycle inhibitor p21 (Figure 2a) and down regulation of c-Myc immunoreactivity an event closely associated with differentiation and inhibition of cycling in leukaemia cells.[15] Furthermore, we observed up-regulation of expression of both the p50 and p60 isoforms of NF-κB, an event closely associated with differentiation of HL60 cells.[16] Direct assessment of CD14 (a cell surface marker for both granulocyte and monocyte differentiation) and CD66b (a marker for granulocyte differentiation only) demonstrated that a 72 hr treatment with violacein induces terminal commitment of the leukemia cells into both lineages at level comparable to DMSO treatment, generally considered a powerful differentiation agent in this model system (Figure 2b). Hence, it is possible that activation of the MAP kinase cassette by violacein produces cell differentiation.

To directly test this hypothesis, we employed the specific p42/p44 MAP kinase inhibitor UO126. In the presence of this inhibitor violacein was no longer capable of inducing differentiation, providing strong support for the notion that activation of the MAP kinase cascade by violacein is required for the induction of differentiation (Figure 2c). Taken together, we feel that increased cell differentiation via MAP kinase activation may contribute to the anti-leukaemic effects of violacein (in addition to the induction of cytotoxicity[13,18]), although final confirmation of this hypothesis will require experiments in patients in which the effect of violacein on leukaemia cell MAP kinase activity and

differentiation are monitored on a single basis. The mechanism by which violacein produces MAP kinase activation remains only partly resolved, the present study shows that it is mediated via activation of the Raf/MEK/MAPK cascade but the signals operating upstream of this cassette are unclear but may well be secondary to the induction of TNF receptor signalling[19]. In that case, the role of TNF receptor signalling in the anti-leukaemic effect of violacein would dichotomic, inducing both direct cytotoxicity via caspase 8 activation and differentiation via activation of MAPK. Disregarding, however, the exact importance for MAP kinase-dependent leukaemia cell differentiation for the anti-leukaemic actin of violacein we feel that the present study shows that kinome profiling using metabolic arrays is a highly promising tool for the rapid evaluation of the effect of pharmacological intervention on signalling pathways.

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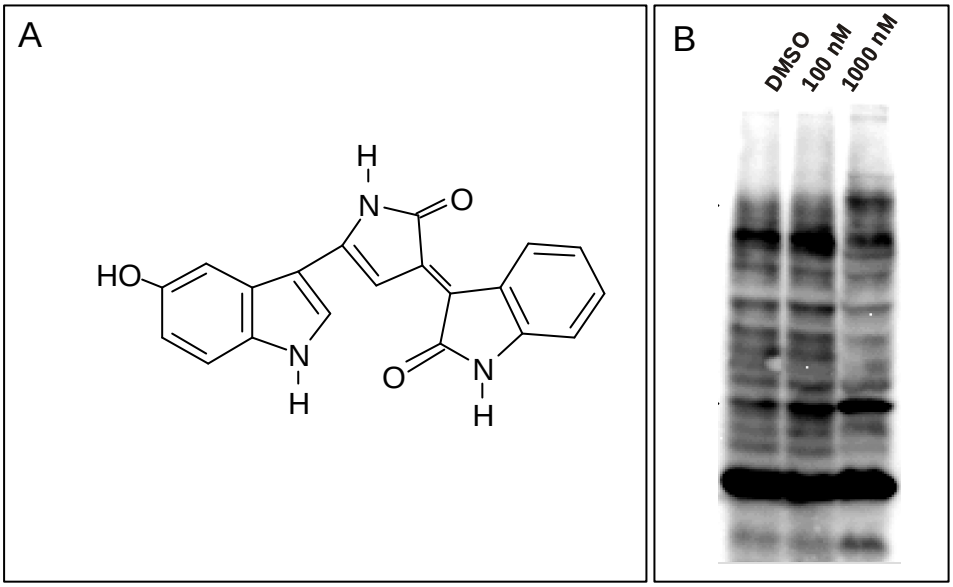

Figure 1. Effects of violacein. A. Chemical structure of violacein. B. HL60 cells were incubated with different concentrations of violacein and subsequently investigated for global phosphorylation patterns employing an anti-pY antibody and Western blotting.

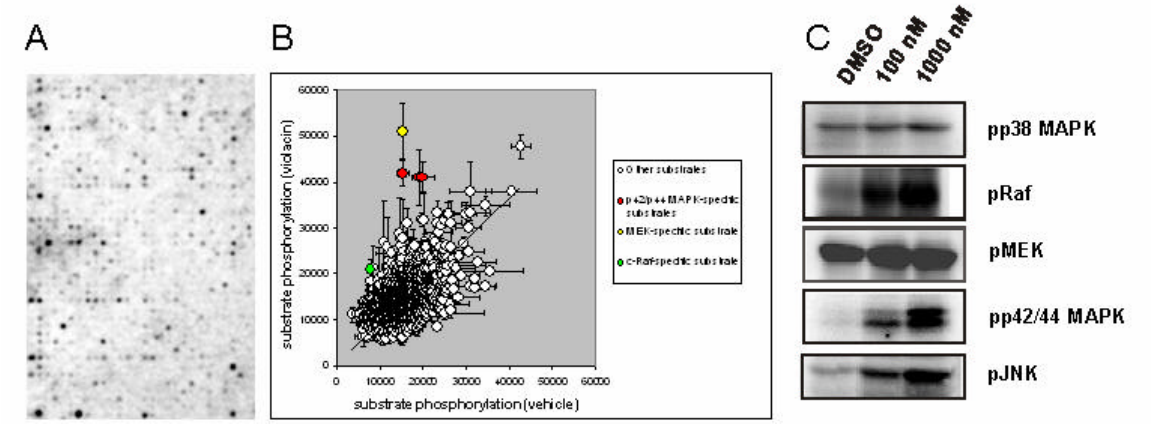

Figure 2. Peptide arrays reveal activation RAF/MEK/MAPK cascade by violacein..A. Example of a peptide array substrate phosphorylation by lysates of HL60 cells treated with 1000 nM violacein. B. The relationship between radio-activity incorporated in substrates phosphorylated by vehicle treated cells against the radio-activity incorporated in substrates phosphorylated by violacein-treated cells. Note the strong violacein-induced effect on the phosphorylation of kipeptides of RAF/MEK/MAPK cascade. C. Analysis of activation of MAPK-associated signalling elements by Western blotting and phosphospecific antibodies.

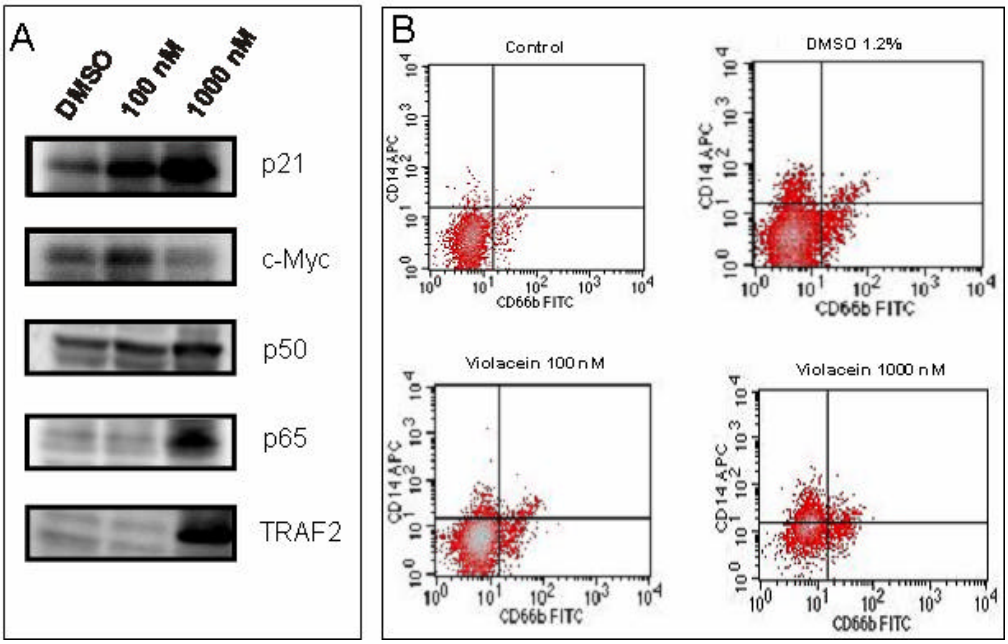

Figure 3. Violacein induces differentiation of HL60 leukaemia cells. a. HL60 cells were incubated with violacein for 24 hrs and the levels of differentiation associated proteins were investigated using Western blotting. b. Direct assessment of HL60 differentiation using FACS and CD14 (a differentiation marker for both granulocytes and monocytes) and CD66b (a marker for granulocyte differentiation only) after 72 hrs of violacein treatment. Treatment with violacein induces terminal commitment of the leukemia cells into both lineages at level comparable to DMSO treatment c. p42/p44 MAP kinase enzymatic activity is essential for violacein-dependent leukaemia differentiation.

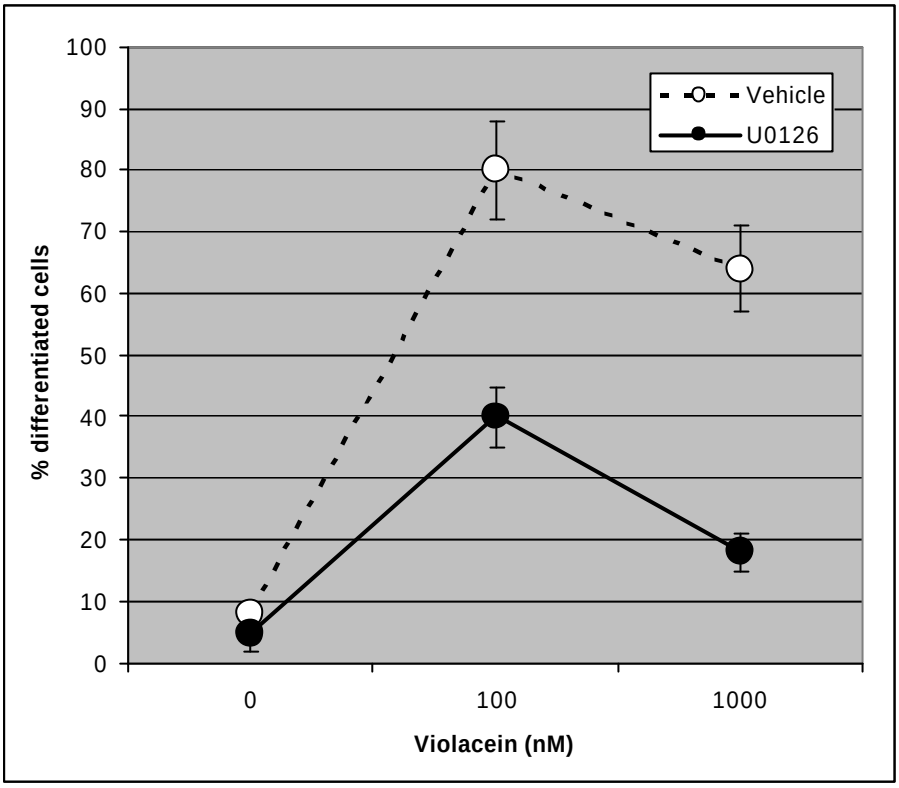

Figure 4. p42/p44 MAP kinase enzymatic activity is essential for violacein-dependent leukaemia differentiation. HL60 cells were incubated with violacein in the presence and absence of 1 ?M of the p42/p44 MAP kinase inhibitor U0126 and investigated for differentiation.